

PHYLOGENY AND BIOGEOGRAPHY OF *EUPATORIUM* (ASTERACEAE: EUPATORIEAE) BASED ON NUCLEAR ITS SEQUENCE DATA¹

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The classification of the predominantly Neotropical Eupatorieae depends upon the circumscription of the core genus *Eupatorium*. The recently proposed narrowing of *Eupatorium* to ~42 species in eastern temperate North America, Europe, and eastern Asia was tested with phylogenetic analysis of nucleotide sequence variation in the internal transcribed spacer (ITS) region of nuclear ribosomal DNA. A total of 40 samples (36 species) of Eupatorieae was analyzed. Several species from North America, South America, and Eurasia that were formerly recognized within a large *Eupatorium* s.l. (sensu lato) were included in the study. Other taxa included were representative of the majority of the subtribes native to eastern temperate North America. Parsimony analysis supported the contention that *Eupatorium* be defined narrowly and suggested that *Eupatoriadelphus* is distinct. The tree topology suggested that *Eupatorium* and *Eupatoriadelphus* share a common North American ancestor with *Liatrix* relative to other Eupatorieae. It was apparent that the presumed sister taxa in Eupatoriinae from South America belong to a different clade. These results suggest that, following initial divergence in North America, *Eupatorium* reached Europe via dispersal during the late Pliocene with subsequent radiation in Asia.

Key words: Asteraceae; biogeography; Eupatorieae; nuclear ribosomal DNA internal transcribed spacer (ITS); phylogeny.

The problems involved with the generic delimitation of *Eupatorium* L. and the related biogeographic issues provide an excellent topic for the application of molecular data. King and Robinson (1987) propose that *Eupatorium* be restricted to 42 herbaceous perennial species that have a north-temperate distribution, four of which have previously been segregated as *Eupatoriadelphus* (Table 1). Although this narrow circumscription has not been universally adopted (e.g., Gleason and Cronquist, 1991; Turner, 1997), recent studies based on chloroplast DNA (cpDNA) restriction site analysis provide support for it (Schilling, Panero, and Cox, 1999). As defined by King and Robinson (1987), the genus exhibits a classic Arcto-Tertiary distribution, with concentrations of species in eastern North America and eastern Asia (King and Robinson, 1970a). The further subdivision of the genus and the phylogenetic and biogeographic relationships of its component units still, however, require clarification, which has not been possible based on morphological data alone.

A combination of morphology and biogeography separate four distinct groups within *Eupatorium* sensu King and Robinson (1987). One of these, "*Eutrochium*," is recognized here as *Eupatoriadelphus* (King and Robinson, 1970b). *Eupatoriadelphus* (*Eupatorium* sect. *Verticillata*; Lamont, 1995) consists of five species, which occur mainly in eastern North America. One of the species, *E. steelei*, was only recently described (Lamont, 1990), and its transfer to *Eupatoriadelphus* is formalized below

(Appendix). Species of *Eupatoriadelphus*, which are also known as "Joe-Pye-weeds," possess nondissected, whorled leaves, and pigmented flowers. "*Uncasia*" is composed of 14 species, which occur primarily in eastern North America (and the West Indies), and is most diverse in the southeastern coastal plain. Species of this group, also known as "bonesets" and "thoroughworts," have nondissected, usually opposite leaves, and nonpigmented (white) flowers. "*Traganthes*" consists of three species, which are most abundant in the coastal plain of southeastern North America. Species of this group, also known as "dogfennels," possess finely dissected, alternate or opposite leaves, and nonpigmented flowers. The Eurasian species, which include the type and sole European species, *E. cannabinum*, have not been recognized formally as an infrageneric taxon (Whittemore, 1987). The Eurasian species have broad, dissected or nondissected, opposite leaves and flowers that may be either pigmented or white.

The relative relationships and taxonomic rank of the groups within *Eupatorium* remain unclear. Studies show that many species may hybridize and that at least some hybrids may form between the "*Traganthes*" and "*Uncasia*" groups (Sullivan, 1978; Jordan, 1991; Sullivan, Neigel, and Miao, 1991). A cytological study (Watanabe et al., 1990) suggests that the Asian and European species may not collectively be monophyletic.

The northern temperate distribution of *Eupatorium* is unique for Eupatorieae, although this distribution is very much like the so-called Arcto-Tertiary distribution of other unrelated temperate genera. Lee et al. (1996) suggest that this pattern of geographic distribution has different possible origins in various taxonomic groups. Phylogenetic analysis of *Eupatorium* and, in particular, elucidation of whether its Eurasian species group is monophyletic, is an important element in the assessment of the

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TABLE 1. List of species of *Eupatorium* and *Eupatoriadelphus* grouped according to species groups or geographic distributions.

Group	Species
<i>Eupatorium</i> s.s.	
North America "Traganthes"	<i>E. capillifolium</i> (Lam.) Small <i>E. compositifolium</i> Walter <i>E. leptophyllum</i> DC.
North America "Uncasia"	<i>E. album</i> L. <i>E. altissimum</i> L. <i>E. cuneifolium</i> Willd. <i>E. hyssopifolium</i> L. <i>E. lancifolium</i> (Torr. & A. Gray) Small <i>E. leucolepis</i> (DC.) Torr. & A. Gray <i>E. mikanioides</i> Chapm. <i>E. mohrii</i> Greene <i>E. perfoliatum</i> L. <i>E. pilosum</i> Walter <i>E. quinqueflorum</i> Urb. & Ekman <i>E. resinosum</i> DC. <i>E. rotundifolium</i> L. <i>E. semiserratum</i> DC. <i>E. serotinum</i> Michx. <i>E. sessilifolium</i> L.
Europe (type species)	<i>E. cannabinum</i> L.
Asia	<i>E. amabile</i> Kitam. <i>E. benguetense</i> C. Robinson <i>E. camiguinense</i> Merr. <i>E. chinense</i> L. <i>E. formosanum</i> Hayata <i>E. fortunei</i> Turcz. <i>E. lindleyanum</i> DC. <i>E. luchuense</i> Nakai <i>E. nodiflorum</i> DC. <i>E. quaternum</i> DC. <i>E. sambucifolium</i> Elmer <i>E. shimadai</i> Kitam. <i>E. squamosum</i> D. Don <i>E. tashiroi</i> Hayata <i>E. toppingianum</i> Elmer <i>E. variabile</i> Makino <i>E. yakushimaense</i> Masam. & Kitam.
<i>Eupatoriadelphus</i>	
North America	<i>E. dubius</i> (Willd. ex Poir.) R. M. King & H. Rob. <i>E. fistulosus</i> (Barratt) R. M. King & H. Rob. <i>E. maculatus</i> (L.) R. M. King & H. Rob. <i>E. purpureus</i> (L.) R. M. King & H. Rob. <i>E. steelei</i> (E. Lamont) G. J. Schmidt & E. E. Schill.

timing and pattern of how *Eupatorium* reached Eurasia. The distribution of the presumed closest relatives of *Eupatorium* is also enigmatic. The subtribal classification provided by King and Robinson (1987) would imply that the closest relatives to *Eupatorium* and *Eupatoriadelphus* (subtribe Eupatoriinae) occur in South America and Africa. The South American Eupatoriinae include *Stomatanthus*, *Hatschbachiella*, and *Austroeupatorium*. In contrast to other genera or subtribes, which range continuously from eastern North America to South America, the Eupatoriinae are virtually absent from Mexico through Central America.

In Asteraceae, ITS (Internal Transcribed Spacer) sequences have proven to be a useful source of information at the generic level to resolve phylogenetic relationships (Baldwin, 1992, 1993; Kim and Jansen, 1994; Sang et al., 1994, 1995; Susanna et al., 1995; Bain and Jansen,

1995; Kim et al., 1996; Schilling and Panero, 1996; Francisco-Ortega et al., 1997; Koopman et al., 1998; Kornkven, Watson, and Estes, 1998; Schilling, Panero, and Cox, 1999; Noyes and Rieseberg, 1999). One advantage in sequencing the ITS region for studies involving a number of species of the same genus is that it is noncoding and so contains a relatively high level of variability. Another advantage to sequencing the ITS region is that it is flanked by highly conserved regions from which universal primers can be obtained (White et al., 1990) and that can serve as reference points for sequence alignment (Baldwin et al., 1995).

The goal of this study was to evaluate the phylogenetic relationships among the members of *Eupatorium* and *Eupatoriadelphus* from eastern North America and Eurasia, and one other representative of the subtribe Eupatoriinae, *Stomatanthus*, using new information from ITS sequence analysis. This would allow further evaluation of the narrowed interpretation of *Eupatorium*, as well as assessment of its biogeography.

MATERIALS AND METHODS

A total of 40 samples of Eupatorieae was examined for ITS sequence variation (Table 2). DNA samples were extracted mainly from fresh leaves using the general CTAB (hexadecyltrimethylammonium bromide) protocol of Doyle and Doyle (1987). The crude DNA extracts of a few samples required further purification using the Wizard Kit protocol (Promega, Madison, Wisconsin, USA). PCR (polymerase chain reaction) reactions were performed in 50- μ L reactions using 10–20 ng of genomic DNA, 10 \times PCR buffer (Promega), 1.8–2.25 mmol/L MgCl₂, 0.2 mmol/L each dNTP, 1.25 units of *Taq* polymerase, and 0.2 μ mol/L each primer. Primers used were "ITS-4" (5'-TCCTCCGCTTATTGATATGC-3') and "ITS-5" (5'-GGAAGTAAAAGTCGTAA-CAAGG-3'; White et al., 1990). PCR was performed with the following protocol: 94°C for two min; 25 cycles of 94°C for 1 min; 52°C for 2 min; 74°C for 1 min; and a final extension of 72°C for 1 min. PCR products were checked by agarose gel electrophoresis and purified by the Wizard Kit protocol. Sequencing was done at the University of Tennessee Automated Sequencing Facility by single-primer PCR with dideoxy terminators, using an ABI sequencer.

ITS sequences initially were aligned with GCG (Wisconsin Package Version 9.0, Genetics Cooperative Group, Madison, Wisconsin) and ClustalX (Thompson et al., 1997) computer programs. Subsequently, the sequences were manually realigned.

Phylogenetic relationships were analyzed based on ITS data using the maximum parsimony approach, implemented with the computer program PAUP*4.0 (Swofford, 1998). Heuristic searches were performed with random sequence addition with replicates to increase the probability that all the shortest trees were found. Gaps were treated as missing data. Bootstrap analysis (Felsenstein, 1985) was performed with PAUP*4.0, using five random addition replicates for each of 1000 bootstrap replicates with the maximum number of trees set at 50. Decay indices (Donoghue et al., 1992) were calculated for each clade of the shortest tree through the use of (converse) constraint trees.

Outgroup taxa were selected from the Helenieae and the Heliantheae s.s. (Heliantheae s.l.), because molecular and morphological studies indicate that these are closely related to Eupatorieae (Jansen et al. 1990; Bremer et al., 1994; Kim and Jansen, 1995). DNA sequences from 12 genera of Heliantheae s.l. were acquired from Genbank, including *Arnica*, *Bahia*, *Dubautia*, *Flourensia*, *Helianthus*, *Heliomeris*, *Madia*, *Perityle*, *Raillardioopsis*, and *Viguiera* (Table 3).

Estimates of divergence times for selected lineages were based upon extrapolation from another set of molecular data (Kim and Jansen, 1995) calibrated with fossil evidence (Graham, 1996). The initial date

TABLE 2. Samples of Eupatorieae analyzed for ITS DNA sequence. The vouchers are in the following herbaria according to collector: LSU: RDT (R. D. Thomas); TENN: PBC (P. B. Cox), EES (E. E. Schilling), GJS (G. J. Schmidt), H. I. (H. Irwin); TEX: JLP (J. L. Panero).

Subtribe	Taxon	Origin	Collector	Genbank accession numbers (ITS1; ITS2) ^a
Oxylobinae	<i>Ageratina altissima</i> -I	Louisiana	RDT 143,177	GBAN-AF177780; GBAN-AF177820
	<i>Ageratina altissima</i> -II	New York	EES 95-17	GBAN-AF177782; GBAN-AF177822
	<i>Ageratina aromatica</i>	Georgia	GJS fb1997-07	GBAN-AF177779; GBAN-AF177819
	<i>Ageratina luciae-brauniae</i>	Tennessee	EES 95-15	GBAN-AF177781; GBAN-AF177821
Mikaniinae	<i>Mikania scandens</i>	Louisiana	RDT 134,183	GBAN-AF177783; GBAN-AF177823
	<i>Brickellia eupatorioides</i>	Tennessee	EES 95-14	GBAN-AF177784; GBAN-AF177824
Alomiinae	<i>Brickellia grandiflora</i>	New Mexico	EES OS274	GBAN-AF177785; GBAN-AF177825
	<i>Chromolaena sagittata</i>	Mexico	HLP 6178	GBAN-AF177786; GBAN-AF177826
Praxelinae	<i>Fleischmannia incarnata</i>	Tennessee	EES 95-21	GBAN-AF177788; GBAN-AF177828
Fleischmanniinae	<i>Ageratum houstonianum</i>	Cult.	EES 95-2	GBAN-AF177789; GBAN-AF177829
Ageratinae	<i>Conoclinium coelestinum</i>	Louisiana	RDT 143,186	GBAN-AF177790; GBAN-AF177830
Gyptidinae	<i>Carphephorus corymbosus</i>	Florida	PBC 5465	GBAN-AF177791; GBAN-AF177831
	<i>Carphephorus pseudoliatris</i>	Florida	PBC 5463	GBAN-AF177792; GBAN-AF177832
Liatrinae	<i>Liatris acidota</i>	Louisiana	PBC 5491	GBAN-AF177795; GBAN-AF177835
	<i>Liatris elegans</i>	Georgia	PBC 5490	GBAN-AF177794; GBAN-AF177834
	<i>Liatris provincialis</i>	Florida	PBC 5473	GBAN-AF177793; GBAN-AF177833
	<i>Trilisa odoratissima</i>	Florida	PBC 5471	GBAN-AF177796; GBAN-AF177836
	<i>Trilisa paniculata</i>	Florida	PBC 5466	GBAN-AF177797; GBAN-AF177837
	<i>Eupatoriadelphus dubius</i>	North Carolina	EES 95-4	GBAN-AF177799; GBAN-AF177839
	<i>Eupatoriadelphus fistulosus</i>	Louisiana	RDT 183,189	GBAN-AF177800; GBAN-AF177840
	<i>Eupatoriadelphus maculatus</i>	New York	EES 95-16	GBAN-AF177798; GBAN-AF177838
	<i>Eupatoriadelphus purpureus</i>	Tennessee	EES 95-11	GBAN-AF177802; GBAN-AF177842
	<i>Eupatoriadelphus steelei</i>	Tennessee	EES 95-7	GBAN-AF177801; GBAN-AF177841
Eupatoriinae	<i>Eupatorium album</i>	Georgia	GJS fb1997-02	GBAN-AF177816; GBAN-AF177856
	<i>Eupatorium altissimum</i>	Tennessee	GJS fb1997-11	GBAN-AF177811; GBAN-AF177851
	<i>Eupatorium cannabinum</i> -I	Cult.*	GJS ecan598	GBAN-AF177806; GBAN-AF177846
	<i>Eupatorium cannabinum</i> -II	Cult.*	GJS ecan716	GBAN-AF177805; GBAN-AF177845
	<i>Eupatorium capillifolium</i> -I	Louisiana	RDT 183,189	GBAN-AF177803; GBAN-AF177843
	<i>Eupatorium capillifolium</i> -II	Cult.*	GJS ecap712	GBAN-AF177804; GBAN-AF177844
	<i>Eupatorium chinense</i> -I	Cult.**	none	GBAN-AF177807; GBAN-AF177847
	<i>Eupatorium chinense</i> -II	Cult.*	GJS echi713	GBAN-AF177808; GBAN-AF177848
	<i>Eupatorium cuneifolium</i>	Georgia	GJS fb1997-01	GBAN-AF177810; GBAN-AF177850
	<i>Eupatorium hyssopifolium</i>	Tennessee	EES 95-9	GBAN-AF177812; GBAN-AF177852
	<i>Eupatorium leucolepis</i>	Georgia	GJS fb1997-03	GBAN-AF177817; GBAN-AF177857
	<i>Eupatorium mikanioides</i>	Florida	GJS fb1997-05	GBAN-AF177818; GBAN-AF177858
	<i>Eupatorium perfoliatum</i>	Tennessee	RDT 143,187	GBAN-AF177814; GBAN-AF177854
	<i>Eupatorium rotundifolium</i>	Georgia	GJS fb1997-06	GBAN-AF177815; GBAN-AF177855
	<i>Eupatorium sessilifolium</i>	Tennessee	EES 95-5	GBAN-AF177813; GBAN-AF177853
	<i>Eupatorium</i> sp. (China)	Cult.*	GJS echi715	GBAN-AF177809; GBAN-AF177849
	<i>Stomatanthes dyctiophyllus</i>	Brazil	HI 8118	GBAN-AF177787; GBAN-AF177827

^a The prefix GBAN- has been added to link the online version of *American Journal of Botany* to GenBank, but is not part of the actual accession number.

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for calibration was an estimate of 40 million years (Ma) for the earliest Asteraceae, based on fossil evidence of Mutiseae-like pollen in South America of 35.4–42.1 Ma (Graham, 1996). To bridge the ITS data with the basal Asteraceae, chloroplast-encoded NADH dehydrogenase (*ndhF*) DNA sequences from 13 species published in Kim and Jansen (1995) were retrieved from GenBank (Table 3). In the *ndhF* data set, the Eupatorieae are derived from within an unresolved clade within Heliantheae. Thirteen Asteraceae taxa including two Eupatorieae taxa were used to estimate the time of divergence between Heliantheae and Eupatorieae lineages. A distance matrix was produced with DNADIST, a part of the Phylip 3.5 software package (Felsenstein, 1993). The Jukes-Cantor model of DNA substitution was used to transform the distance matrix to compensate for multiple substitution, with transitions treated equally to transversions. Because the closest relative to the Eupatorieae is uncertain, the sequence divergences between the Eupatorieae and five Heliantheae sequences were averaged. This distance was divided by the average divergence between *Barnadesia* (part of a group that diverged very early in the family; Jansen and Palmer, 1987), and the remaining Asteraceae; this ratio was multiplied by the assumed 40-

Ma age of the Asteraceae to give the age of the Eupatorieae-Heliantheae split. For the ITS sequence data, the average distance between the *Ageratina-Mikania* clade and the remaining Eupatorieae was divided by the average distance between the Eupatorieae and the Heliantheae taxa in the ITS data set; this ratio was multiplied by the age of the Eupatorieae-Heliantheae split determined from the *ndhF* data set.

The date estimates for the divergence of taxa within the Eupatorieae were made by a system of nested branch length ratios calculated from neighbor-joining trees produced with the ClustalX software (positions with gaps were excluded; Thompson et al., 1997). The reference date was the divergence between the *Ageratina-Mikania* clade and the remaining Eupatorieae as calculated above. Calculations were made from a neighbor-joining tree in preference to individual pairwise comparisons, because adjustments for unequal rates of evolution could be made. Neighbor-joining branch lengths were used in preference to cladograms because: (1) compensation can be made for the undetected multiple substitutions occurring between taxa; and (2) ambiguous character-state changes are not factors in distance-based methods. In calculating ages from neighbor-joining trees, it was not assumed that mutation rates were

TABLE 3. GenBank accession numbers for *ndhF* sequences used to calibrate the molecular clock and ITS sequences used as the outgroup.

Species	Gene	GenBank accession number
<i>Aster cordifolius</i>	<i>ndhF</i>	GBAN-L39449
<i>Bahia absinthifolia</i>	<i>ndhF</i>	GBAN-L39464
<i>Barnadesia caryophylla</i>	<i>ndhF</i>	GBAN-L39394
<i>Carthamus tinctorius</i>	<i>ndhF</i>	GBAN-L39417
<i>Chromolaena</i> sp.	<i>ndhF</i>	GBAN-L39375
<i>Coreopsis tinctoria</i>	<i>ndhF</i>	GBAN-L39461
<i>Eupatorium atrorubens</i> = <i>Bartlettina sordida</i>	<i>ndhF</i>	GBAN-L39376
<i>Flaveria ramosissima</i>	<i>ndhF</i>	GBAN-L39465
<i>Geraea canescens</i>	<i>ndhF</i>	GBAN-L39381
<i>Gerbera jamesonii</i>	<i>ndhF</i>	GBAN-L39403
<i>Helianthus annuus</i>	<i>ndhF</i>	GBAN-L39383
<i>Venegasia carpesioides</i>	<i>ndhF</i>	GBAN-L39379
<i>Verbesina robinsonii</i>	<i>ndhF</i>	GBAN-L39377
<i>Vernonia mespilifolia</i>	<i>ndhF</i>	GBAN-L39427
<i>Argyroxiphium caliginis</i>	ITS	GBAN-M93788
<i>Arnica mollis</i>	ITS	GBAN-M93789
<i>Bahia absinthifolia</i>	ITS	Baldwin, unpublished data
<i>Dubautia latifolia</i>	ITS	GBAN-AF061900
<i>Flourensia monticola</i>	ITS	Schilling & Panero, 1996
<i>Helianthus divaricatus</i>	ITS	GBAN-AF047954
<i>Heliomeris multiflora</i>	ITS	Schilling & Panero, 1996
<i>Hymenopappus filifolius</i>	ITS	Baldwin, unpublished data
<i>Madia elegans</i>	ITS	GBAN-M93795
<i>Perityle megacephala</i>	ITS	Baldwin, unpublished data
<i>Raillardopsis scabrida</i>	ITS	GBAN-M93799
<i>Viguiera tomentosa</i>	ITS	Schilling & Panero, 1996

constant throughout the phylogeny. But it was assumed that mutation rate is locally consistent between nodes, so that the average mutation rate is comparable between branches and their connecting stems. This method compensates for variation in mutation rates between lineages and between nodes. The first step in age estimation involved averaging terminal branch pairs. A ratio value was calculated between value branch averages and the sum of the length of the stem connecting the branches and the branch average. Each sum of stem length and branch average was treated as a total branch length, and was then averaged with the total branch length of the adjacent lineage. Each ratio between average branch length and total branch length was multiplied by the reference date and the ratio values of every ancestral node below it to the reference node. Thus, at each node the "molecular clock" was allowed to be "reset" for a different mutation rate. The mutation rate per million years was calculated for each node of the neighbor-joining tree by dividing the sum of two averaged branches diverging at the node by the age of the node.

Tests were made for differences in rates of molecular evolution among several pairs of taxa using the method of Tajima (1993). This method involves the comparison of two sequences relative to a third (outgroup) sequence. To perform this test, all characters showing a gap in at least one taxon were eliminated. For each pair of sequences, every position was counted for which only one of the pair differed from the outgroup sequence. The difference in the number of informative changes in the sequence pair was squared and divided by the sum of those changes. The quotient was then rated within the chi-square distribution with 1 df and a 5% probability value. The transversal changes (pyrimidines, C or T, to or from purines, A or G) and transitional changes (pyrimidines to pyrimidines and purines to purines) were partitioned, and their quotients were added together. They were also rated within a chi-square distribution (2 df).

RESULTS

ITS sequences—The length of the ITS region in *Eupatorium* was similar to lengths reported for the Helian-

theae (Schilling and Panero, 1996). The sequenced region was 631–652 base pairs (bp) in length. ITS-1 was 258 bp (range = 257–260 bp). The 5.8S region was uniformly 164 bp. ITS-2 was 213 bp (range = 204–231 bp) for *Eupatorium* and 228 bp in *Eupatoriadelphus*; the differences in ITS-2 size reflect the presence of gaps of 11 and 3 bp, respectively, for the two genera.

To align the sequences, the insertion of gaps required an increase in the total number of characters in the data set by ~20% in both ITS-1 and ITS-2. The total number of gaps required to align the 52 taxa was 47 and 40 for ITS-1 and ITS-2, respectively. The total length of the character matrix was 563 characters. The number of characters showing no variation was 72 (24%) and 71 (28%) for ITS-1 and ITS-2, respectively. The number of characters that varied in more than one taxon, and thus were parsimoniously informative, was 187 (61%) and 131 (51%) for ITS-1 and ITS-2, respectively. Altogether, there were 420 (75%) polymorphic positions and 318 (56%) informative characters.

The level of sequence divergence between the outgroup (e.g., *Helianthus divaricatus*) and ingroup taxa (Eupatorieae) varied from 110 to 140 bases, or from 26 to 30% (35 to 40% with correction for multiple substitutions). The most distant lineages in the Eupatorieae relative to *Eupatorium*, *Mikania*, and *Ageratina*, had 17–27% divergence relative to the closer lineages (23%–36% with corrections for multiple-base substitutions). The five *Eupatoriadelphus* species were 1–21 bases (<1 to 5%) divergent from each other and 33–61 bases (8–16%) divergent from *Eupatorium*. Divergence among samples of *Eupatorium* was 2–43 bases (<1 to 10%).

Phylogenetic analysis—Wagner parsimony analysis of the 40 taxon/563 (370 without gaps) character matrix with 12 Heliantheae as the outgroup resulted in 24 equally parsimonious trees of 1368 steps in length with a consistency index of 0.49 (0.47 excluding uninformative characters), a retention index of 0.75, and a rescaled consistency index of 0.37 (Fig. 1). The 24 most parsimonious trees differed only in the placement of some "*Uncasia*" species, *Eupatoriadelphus* species, and *Ageratina* samples. *Mikania* and *Ageratina* formed a sister clade to the rest of the Eupatorieae. *Brickellia* ($x = 9$) formed a sister clade to the rest of the $x = 10$ taxa. *Conoclinium*, *Ageratum*, and *Fleischmannia* comprised a sister clade to a clade containing *Chromolaena* and *Stomatanthes*. The *Chromolaena-Conoclinium* clade was sister to the two clades of Liatrineae and *Eupatorium* + *Eupatoriadelphus*. *Liatris* was monophyletic, whereas *Carphephorus pseudoliatris* was basal to *Liatris*; the remaining *Trilisa* and *Carphephorus* taxa formed a monophyletic trichotomy due to the lack of variation among them. Within the *Eupatorium* clade, *E. cuneifolium* was sister to the rest of the genus. Except for *Eupatorium cuneifolium*, the "*Uncasia*" samples formed a sister clade to the Eurasian taxa.

Clades within the ITS tree (Fig. 1) received various levels of support. The clade that included *Eupatorium* + *Eupatoriadelphus* was only weakly supported, with a decay index of 2 and a bootstrap value of only 47.2%, whereas the *Eupatorium* and *Eupatoriadelphus* clades were each strongly supported, with bootstrap values of 99.9 and 94.9%, respectively, and decay indices of 17

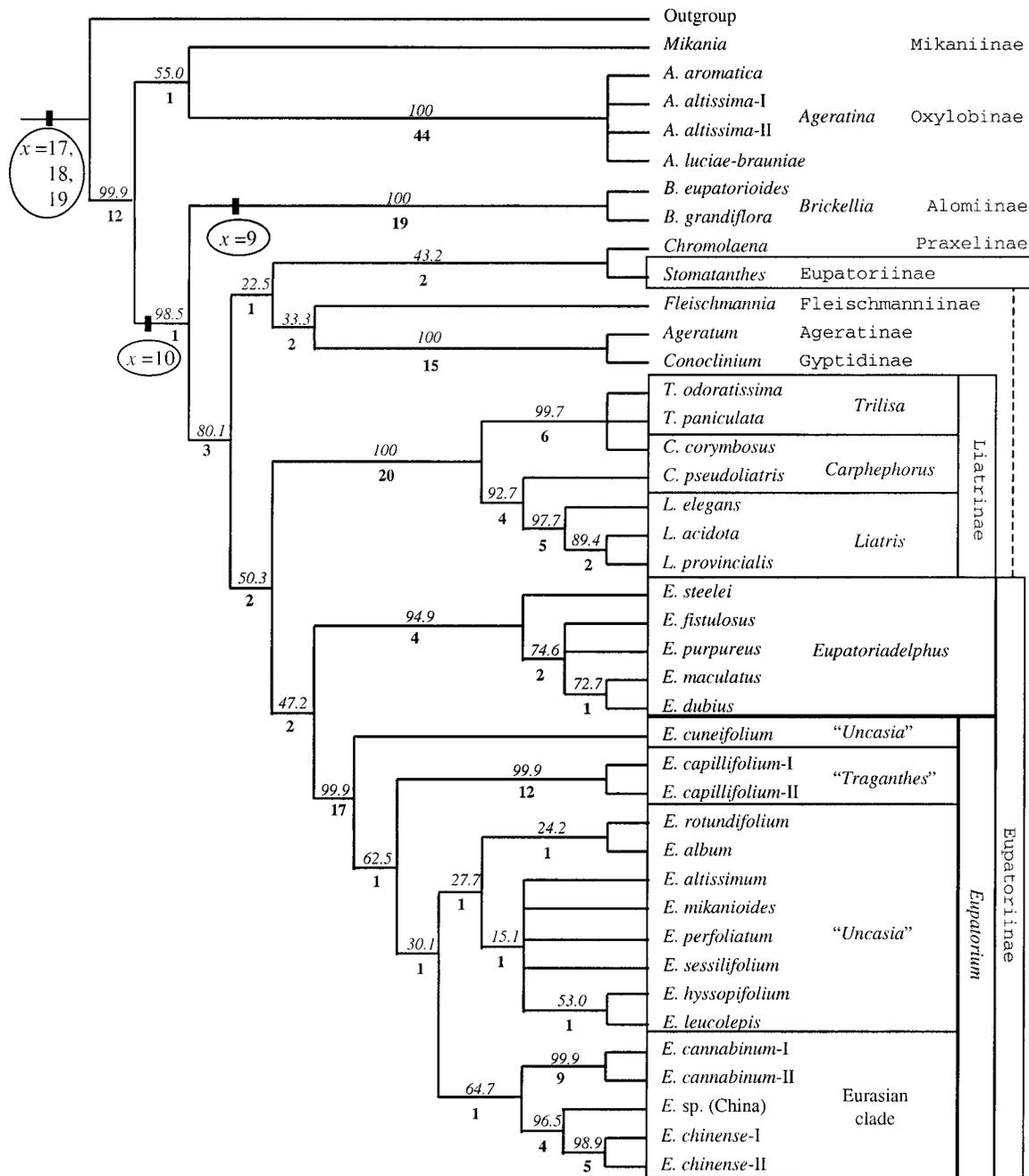


Fig. 1. Strict consensus of 24 equally parsimonious trees showing the relationships among samples of Eupatorieae based on ITS DNA sequence data. Bootstrap analysis results (percentage of 1000 replicates) are shown above each branch; decay indices are shown below each branch. Base chromosome number is indicated on each clade for which it changes.

and 4, respectively (Fig. 1). The *Eupatorium* clade was independently supported by the presence of an 11-bp synapomorphic deletion. The *Eupatoriadelphus* clade was independently supported by the presence of a 3-bp insertion. The *Eupatorium* s.s. (sensu stricto) clade included the three groups of *Eupatorium*, “*Traganthes*,” “*Uncasia*,” and the Eurasian species. Clades within “*Uncasia*” were only weakly resolved with very low bootstrap values and decay indices of zero to one. Members of the Liatrinae (*Liatris*, *Trilisa*, and *Carphephorus*) formed a very strongly supported clade (bootstrap value of 100%

and decay index of 20), which was sister to *Eupatorium* s.l. with moderately low support (bootstrap value of 50.3% and decay index of 2). The relationship of Liatrinae and *Eupatorium* + *Eupatoriadelphus* was supported by four unambiguous character state changes, or seven changes total under the accelerated transformation (ACCTRAN) option in PAUP*4.0 (Fig. 2), one of which was within the 11-base gap within *Eupatorium*.

Date estimation and evolutionary rates—Analysis of the subset of the *ndhF* sequence data of Kim and Jansen

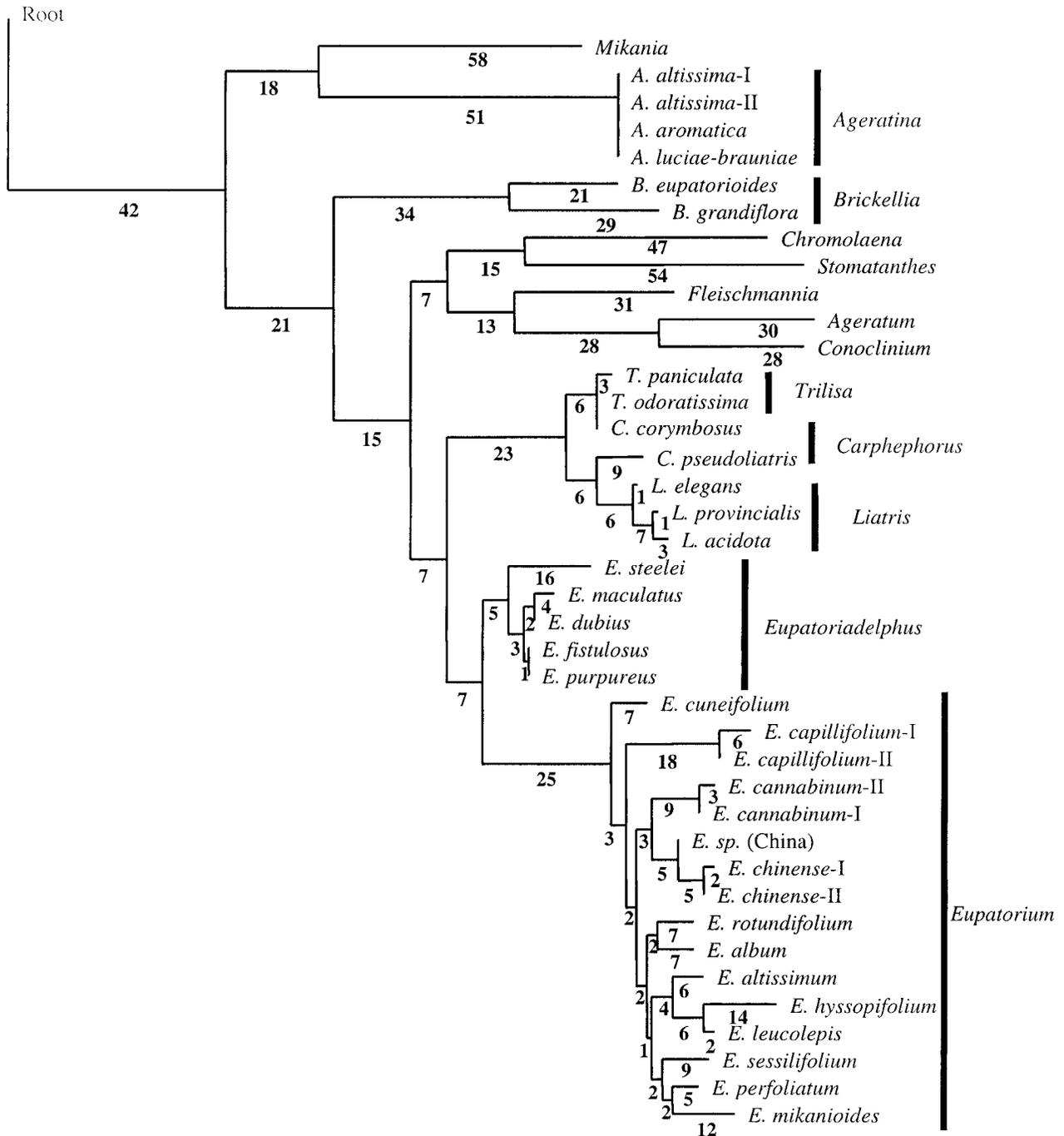


Fig. 2. One of 24 equally parsimonious trees (compatible with neighbor joining tree constrained with consensus tree) showing the relationship of samples of Eupatorieae, based on ITS DNA sequence data. Numbers of nucleotide substitutions are shown below. Branch lengths were calculated with the accelerated transformation (ACCTRAN) option in PAUP*4.0.

(1995) relative to the estimated 40 Ma for the first emergence of the Asteraceae suggested that the time of divergence between the Eupatorieae and the Heliantheae outgroup taxa was 14.8 Ma. Allowance for variance in mutation rates or differences in relationships yielded a minimum age of 10.9 Ma if only *Venegasia carpesioides* was used and a maximum age of 19.7 Ma if only *Helianthus annuus* was used.

With an average (corrected) ITS sequence divergence

of 34.6% between the Heliantheae and the Eupatorieae, a 14.8-Ma divergence time implies a mutation rate of 2.34% per million years (range of 2.27–2.53%/Ma). The average time of divergence between the *Ageratina-Mikania* clade and the remaining taxa in the Eupatorieae clade was estimated to be 12.1 Ma. When gaps occurring in more than three taxa were removed from the data set, the resultant time estimates were almost the same (11.9 Ma was the average divergence for the basalmost node

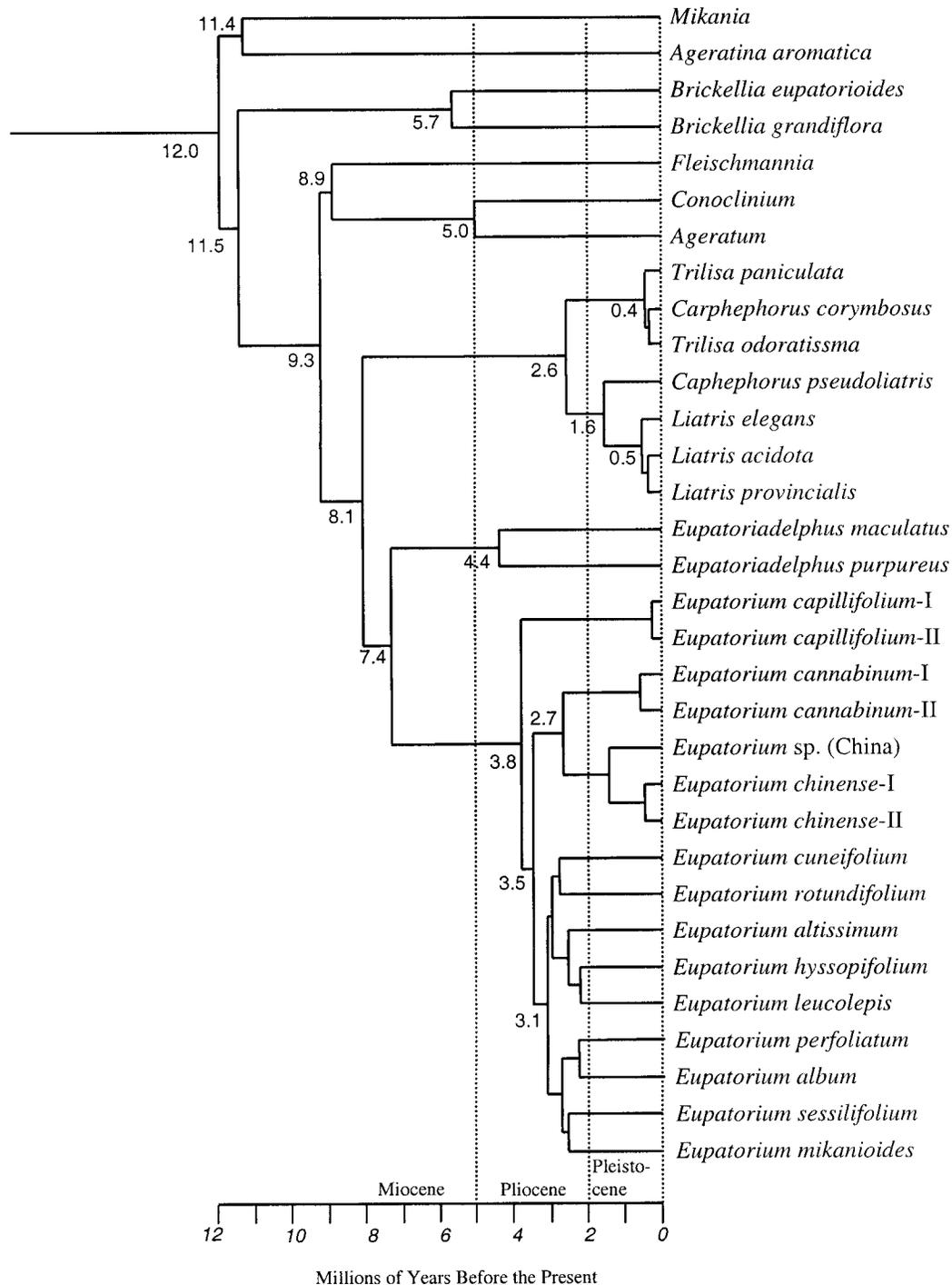


Fig. 3. Estimated time of divergence of Eupatorieae species based upon ITS DNA sequences and neighbor-joining branch length averaging (Schmidt, 1998). Numbers subtending nodes indicate the millions of years (Ma) preceding the present ($\pm 35\%$).

in the Eupatorieae). The minimum and maximum values for this divergence time were calculated as 8.2 and 16.6 Ma, respectively, when the extremes from the *ndhF* data and the ITS data were combined.

The neighbor-joining tree dates were calibrated with the 12-Ma divergence time for the outermost node of the Eupatorieae (Fig. 3). Because the actual value was calculated to be anywhere from 8.2 to 16.6 Ma, each date

calculated from the neighbor-joining tree can be assumed to have a margin of error of 35%. The divergence times between the *Eupatorium* and the *Brickellia*, the *Ageratum*, the *Liatrinae*, and the *Eupatoriadelphus* clades were calculated to be 11.5, 9.3, 8.1, and 7.4 Ma, respectively. The divergence time between the “*Traganthes*” group and the rest of *Eupatorium* was 3.8 Ma. The time of the split between Eurasian and North American *Eupatorium*

was estimated to be 3.5 Ma. The divergence between European and Asian *Eupatorium* was estimated to be 2.7 Ma. The estimated divergence among species of the “*Uncasia*” group was from 2.2 to 3.1 Ma.

The mutation rates implied from the date estimates were generally ~2%/Ma, but within some lineages, the rate had decreased to as low as 1.06%/Ma (*Eupatoriadelphus*) or increased to nearly 4%/Ma (some terminal lineages in *Eupatorium* and *Liatrinae*). An intermediate mutation rate of 1.95%/Ma was estimated at the basal-most node of *Eupatorium*.

Rate heterogeneity was found to be significant in two out of 13 pairwise comparisons of the ITS sequences at the $\alpha = 0.05$ level (Schmidt, 1998). With *Helianthus* as the outgroup, the mutation rate for *Ageratina* was not significantly higher than *Eupatorium* except when transversions were treated independently of transitions. However, regardless of the type of mutation, *Eupatoriadelphus* had a significantly different mutation rate relative to *Eupatorium* when *Ageratina* was used as the outgroup taxon. Thus, a molecular clock could not be assumed if *Eupatoriadelphus* and *Ageratina* were left in the data set.

Because the *Eupatoriadelphus* clade deviated in mutation rate more than most other clades in the neighboring tree, a recalculation of dates was made without *Eupatoriadelphus* present. Date estimations made without *Eupatoriadelphus* were not radically different from those made initially. The age of divergence for *Eupatoriadelphus* and *Eupatorium* was 7.6 Ma, when *Eupatoriadelphus* was removed (rather than 7.3 Ma). The age of every node within *Eupatorium* was <5% higher than before, whereas the ages of the *Liatrinae* were only 2% higher, and the ages below these clades were <1% higher. Therefore, the most important source of error was the approximate $\pm 35\%$ from bridging the ITS data with the *ndhF* data, rather than from rate variation within the Eupatorieae.

DISCUSSION

The ITS sequence derived phylogeny of Eupatorieae showed a dispersion of genera traditionally placed in *Eupatorium* to other clades (*Ageratina*, *Conoclinium*, *Fleischmannia*), providing a further indication that *Eupatorium* s.l. is an artificial concept. *Eupatorium* sensu King and Robinson (1987) occurred in two well-supported monophyletic groups within *Eupatorium*, which together formed a more weakly supported monophyletic group. This phylogeny was not in conflict with either King and Robinson (1970b, 1987) concept of *Eupatorium*. The lack of support for branches within the genus may be a hint that the radiation of the modern lineages from a single common ancestor was relatively rapid.

***Eupatorium* s.l. an artificial concept**—The ITS sequence data strongly supported the segregation of *Ageratina*, *Conoclinium*, and *Fleischmannia* from *Eupatorium*. The placement of *Ageratina* with *Mikania* outside the remaining Eupatorieae taxa is compatible with the chloroplast restriction site data, which places *Ageratina* and *Mikania* in an unresolved polytomy with other genera of high base chromosome number of $x = 17, 18,$ or 19 at the base of the Eupatorieae clade (Schilling, Panero,

and Cox, 1999; Tetsukasu Yahara, personal communication). This is not too surprising, because the higher chromosome count is considered by Bremer et al. (1994) and Watanabe et al. (1995) to be a plesiomorphy for the tribe, with subsequent reduction in base chromosome number to $x = 9$ or 10 in the more derived sections of the tribe. Somewhat surprisingly, *Ageratum*, a genus that is distinguished from *Eupatorium* s.l. by its pappus of scales, was the sister taxon to *Conoclinium*, which possesses a pappus of capillary bristles; this close relationship suggests that the pappus in *Ageratum* may merely represent a recently derived state rather than an indication of a fundamental split. *Fleischmannia* also fell within the *Ageratum* clade, although not with as much support. Such a relationship between these groups is not suggested by the subtribal classification of King and Robinson (1987). This result was, however, congruent with trees derived from cpDNA restriction site data (Schilling et al., 1999). The implication of these results is that the traditionally defined *Eupatorium* s.l. is based mainly on a set of symplesiomorphies found throughout the tribe. Genera that are traditionally outside *Eupatorium* s.l. are distinguished by one or more easily seen, derived features (e.g., pappus of scales in *Ageratum*; ten-angled achenes in *Brickellia*; and alternate leaves, spike-like inflorescence, and ten-angled achenes in *Liatris*). The retention of *Ageratina*, *Conoclinium*, and *Fleischmannia* in *Eupatorium* would, however, require the inclusion of such genera as *Brickellia*, *Ageratum*, and *Liatris* for the genus to be monophyletic. Clearly, *Eupatorium* as circumscribed by King and Robinson (1987) is a significant improvement.

***Eupatoriadelphus* as a genus**—The ITS sequence derived phylogeny of the Eupatorieae distinguished *Eupatoriadelphus* and *Eupatorium* as two well-supported clades. Reliable morphological differences exist to support these two clades (Schmidt, 1998). In contrast, few molecular synapomorphies exist to support the combined *Eupatoriadelphus*-*Eupatorium* clade. The combination of these results favors a recognition of *Eupatoriadelphus* as a genus distinct from *Eupatorium*.

Status of “*Uncasia*,” “*Traganthes*,” and Eurasian species—Based on the ITS sequence data, there was little resolution among the species of *Eupatorium*. At most, it may be concluded that “*Traganthes*” and the Eurasian taxa were derived from a paraphyletic “*Uncasia*,” which is consistent with the results of Schilling, Panero, and Cox (in press); formal recognition of the groups does not appear to be warranted.

Subtribal classification—The Eupatoriinae as defined by King and Robinson (1987) include, in addition to the temperate *Eupatorium* (and *Eupatoriadelphus*), the mainly South American *Stomatanthes*, *Hatschbachiella*, and *Austroeupatorium*. In this study, however, at least one member of the South American group, *Stomatanthes*, appeared to be more closely related to Praxelinae (*Chromolaena*) than it did to any member of the *Liatrinae*-*Eupatorium* clade. Thus, the subtribal classification of King and Robinson (1987) is not consistent with the molecular phylogeny.

Sequence divergence and mutation rates—The earliest fossil record for the Asteraceae consists mainly of pollen. Bremer and Gustafsson (1997) use the Eocene-Oligocene boundary of 38 Ma for the age of the Asteraceae based upon fossil references. They also use the separation of South America and Antarctica of 43–53 Ma to date the divergence between Australian Goodeniaceae and South American Calyceraceae, which are relatives of the Asteraceae. The estimated rates of substitution for *rbcL* derived from either of these calibrations (Bremer and Gustafsson, 1997) are almost exactly the same. This provides verification for the calibration of the *ndhF* data with the estimation of 40 Ma as the age of Asteraceae.

The mutation rates estimated for the Eupatorieae ITS region (1–3%/Ma) were in general very high relative to rates reported for this gene region in other plant groups. The Cucurbitaceae, a family of herbaceous annuals and perennials, are estimated to have at most a rate of 0.362%/Ma (Jobst, King, and Hemleben, 1998). Such a mutation rate applied to the Eupatorieae would imply that the tribe was almost 80 million years old, or twice as old as the Asteraceae fossil record. An example of mutation rates from the Asteraceae can be found for the Hawaiian silverswords (tribe Heliantheae s.l.), which are shrubs and monocarpic perennials. Of the silverswords, the age of divergence between *Dubautia* and *Argyroxiphium* is estimated to be 5.2 Ma (Baldwin and Sanderson, 1998). Two sequences from these genera (Table 3) showed a divergence of 0.700%/Ma (according to the age estimates used in Baldwin and Sanderson, 1998). This is still less than half the rate encountered in the Eupatorieae. In general, life history traits, such as shorter generation time, may partially explain higher mutation rates in the ITS region (Baldwin et al., 1995).

Biogeography of Eupatorium—As currently defined, the New World range of the Eupatoriinae is characterized by a lack of continuity through Mexico and Central America. One North American member of the Eupatoriinae (*Eupatorium serotinum*) occurs as far south as Mexico near the Texas border, whereas South American Eupatoriinae (*Austroeupatorium inulaefolium*) only reach as far north as Panama (King and Robinson, 1987). This contrasts with the distribution of other subtribes represented in eastern North America (Mikaniinae, Fleischmanniinae, Oxylobinae, and Alomiinae), which are represented more or less continuously to South America. With the discovery that *Stomatanthes* was not monophyletic with the North American Eupatoriinae according to the ITS data, the geographic disjunction no longer requires explanation.

A novel result of this study was the close relationship of the Liatrinae and the temperate Eupatoriinae. Although this relationship is unresolved in Schilling et al. (1999) and was only weakly supported by the ITS data, their common geographic range does suggest that both groups radiated in the southeastern coastal plain of the United States. The centers of diversity of both *Eupatorium* and Liatrinae are in Florida. Like the temperate Eupatoriinae, the Liatrinae occur almost exclusively east of the Rocky Mountains north of Mexico. The time of divergence between these clades was estimated to be ~8 Ma, during the late Miocene. By this time, the cordillera had effec-

tively separated eastern and western floras of North America (Graham, 1993). The forests in the Great Plains region gradually developed into savanna as the Rocky Mountain rain shadow strengthened. Such an environment of forest edges may have been conducive to the northward extension of a *Eupatorium*-Liatrinae ancestor.

Although the distribution of *Eupatorium* in Eurasia and North America is unique among Eupatorieae, this pattern is not uncommon among genera in many other families. The similarity between the floras of eastern Asia and eastern North America has long been noted (Takhtajan, 1986), especially for taxa associated with mesic broadleaf deciduous forest zones, and is attributed to a former connection between the regions during the Tertiary period (Sharp, 1951; Wolfe, 1975; Tiffney, 1985; Graham, 1993). It is presumed that this previously continuous “Arcto-Tertiary Geoflora” was later differentially fragmented to a degree in which relict genera occur endemically in the humid temperate eastern United States, the southern mountains of Mexico, China, or California.

Although *Eupatorium* may fit an “Arcto-Tertiary” pattern (King and Robinson, 1970a), a critical assessment must be made of paleogeography to determine whether its present distribution resulted from range dissection or through long-distance dispersal. The lack of other, related genera (*Eupatoriadelphus*) in Eurasia and the predominantly Neotropical distribution of the Eupatorieae argue for a North American origin for *Eupatorium*. Because it is likely that European and Asian *Eupatorium* are a monophyletic group, only one colonization event needs to be invoked. During the Eocene a contiguous belt of mixed temperate forests spanned the Northern Hemisphere. These continents reconnected both via a Bering Sea land bridge and a northern North Atlantic land bridge (Graham, 1993). The circumpolar connection began shortly after the Cretaceous period when the transcontinental seas, which had segregated the eastern and western portions of both North America and Eurasia, receded. The Eocene flora consisted of temperate taxa that likely migrated via the North Atlantic land bridge, until Europe finally completed its separation from North America and Greenland. However, Asteraceae did not become widespread until ~25 Ma ago, during the late Oligocene epoch. Thus, vicariance is an unlikely mechanism for *Eupatorium* to enter Europe.

After the Eocene, the climate cooled, but it was still warm enough to allow an exchange of temperate taxa between North America and Asia via the Bering Sea land bridge not quite up to the Pleistocene (2 Ma ago), when only tundra steppe existed in this region between times of sea inundation (Delcourt and Delcourt, 1993). By the Pliocene, as the climate grew cooler, the deciduous forests were replaced by conifers in Alaska. The forests were replaced later in the Pliocene by a boreal shrub and sedge community. The estimated time of divergence of 2.7 Ma between European and Asian species and 3.5 Ma between North American and Eurasian species of *Eupatorium* would make it likely that boreal conditions existed for these migrants. Both upper and lower age estimates based upon the DNA data for this divergence (2.3–4.7 Ma according to a $\pm 35\%$ error) fall within the Pliocene. With only one contemporary species of *Eupatorium* reaching into the boreal zone (*E. perfoliatum*) it seems

less likely, but not impossible, that the ancestor to the Eurasian species migrated through the Bering Sea land bridge. Perhaps the lack of ice age refugia explains the modern absence of *Eupatorium* in Alaska. However, *Eupatorium* are also presently absent in other portions of western North America, making it doubtful that they have ever been present in the flora of western North America.

The alternative explanation to vicariance is long-distance dispersal. *Eupatorium* species possess achenes capable of wind dispersal typical of many other weedy composites such as *Taraxacum officinale* (Sheldon and Burrows, 1973). When mature, the *Eupatorium* pappus spreads out as water is lost from the cells below the pappus bristles. Cells at the base of the involucre bracts had a similar appearance to those at the pappus base and may operate through similar mechanisms to allow the bracts to open (although the innermost bracts abscise), allowing the wind to capture the achenes. Although the primary adaptation of this dispersal mechanism is probably to move the fruit over relatively short distances to escape from an unsuitable local condition (such as competition or inadequate soil), it could also allow more rarely long-distance dispersal. Europe is the most direct target for long-distance dispersal via prevailing westerly winds. Although the greater diversity of *Eupatorium* in Asia would favor the hypothesis that the genus was there for a longer time than in Europe, the chromosome morphology of the Asian *Eupatorium* is more distinctive than *E. cannabinum* compared to North American species (Watanabe et al., 1995). If the Asian species group is monophyletic, then the simplest migration model is one in which Europe was colonized first, followed by a migration to Asia. The current lack of species diversity in modern Europe is possibly due to the greater susceptibility of European taxa to extinction during Pleistocene climatic oscillations (Liu, 1988).

Conclusions—The results provided further support for a narrow delineation of *Eupatorium*, and for recognition of *Eupatoriadelphus* as distinct, but do not support the subtribal classification of King and Robinson (1987). The amount of DNA sequence divergence, in association with other factors, was used to suggest that the current Northern Hemisphere distribution of the genus was achieved through relatively recent dispersal, rather than through vicariance resulting from exchange in the Tertiary Period.

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APPENDIX. New combination in *Eupatoriadelphus*.

Eupatoriadelphus steelei (Lamont) G. J. Schmidt & E. Schill. *comb. nov.*
 Basionym: *Eupatorium steelei* Lamont (1990) *Brittonia* 42: 279.
